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FULL PAPER

Metagenomic analysis of bacterial community in a travertine depositing hot spring

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SUMMARY

Several factors influence bacteria biodiversity in hot springs. The impact of biotic and abiotic pathways on travertine deposition plays a key role in microbial ecology and in the final composition of the waterborne microbiota. The metabolism of some bacterial groups such as photoautotrophs or lithoautotrophs influences water chemistry, favoring carbonate precipitation processes. The role of microbial mats in mineral precipitation processes is not fully clarified. For the first time, a comprehensive metagenomic analysis has been undertaken in the historical Bullicame hot spring. Bacterial biodiversity was characterized and biomineralization activities were assigned to different genera. A higher biodiversity in mat samples compared to water samples was observed: Shannon index of 3.34 and 0.86, respectively. Based on the functional assignment of each Operational Taxonomic Unit, the bacteria involved in biologically-induced mineralization are prevalent in mat and released in the water. According to the principle that each geothermal water specimen has distinctive physic-chemical characteristics, our results suggest new interacting bio-actions within these ecosystems. The saturation index and the chemical composition, as the high concentration of sulfur species and HCO_3, can be linked to create a selective environment where pioneer communities are able to live and shape the ecosystem.

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INTRODUCTION

Hot springs are natural water environments present all over the world and characterized by specific physical and chemical characteristics, including pH, redox potential and the presence of several trace elements at higher levels than those found in fresh or ground waters (Yazdi, 2015; Gagliano, 2016). These properties directly or indirectly affect the microbial component in hot springs (Mathur *et al.,* 2007). Although temperature, pH and specific chemical components seem to be major factors influencing microbial community profiles, hydrogeological and geographical features also play a significant role (Meyer-Dombard *et al.,* 2005; Gagliano, 2016; Song *et al.,* 2013). The study of biodiversity in these extreme environments represents much more than the mere description of the microorganisms inhabiting extreme habitats. It offers further insight into species interrelations, their impact on geochemical cycles and the influence of physicochemical variables on community structure, providing information on complex relationships between prokaryotes and extreme environmental conditions (Fouke, 2011; Kim *et al.,* 2011).

The Apennine Peninsula is characterized by thousands of hot springs and the area of Viterbo presents renowned volcanic ground waters known since The Etruscan and Roman civilizations and cited by Dante Alighieri (Yuhara, 1963). These waters today are still used for wellness and SPA (Salus Per Aquam) therapy. In this area, there are four main geological zones, including the Bullicame area to the west of Viterbo. This thermal area hydrogeology profile shows numerous hot springs and pools without superficial mingling of waters belonging to a same recharge area, i.e. Cimini Mountains-Lake Vico (Piscopo *et al.,* 2006; Di Salvo, 2013). This region shows a large range of thermal and chemically abnormal values with a maximum temperature of 65°C, a minimum pH of 5 and conductivity about 3000 µS/cm (Di Salvo, 2013). Preliminary data on organic components and microflora are also available (Curri *et al.,* 1997; Seyfried *et al.,* 2002).

Bullicame and other worldwide springs, such as those in Yellowstone National Park, are classified as one of the still active travertine depositing hot springs (Fouke, 2011). In these sites, the impact of biotic and abiotic communities on travertine deposition can play a major role in microbial ecology and waterborne microbiota (Pentecost, 2005; Di Benedetto *et al.,* 2011). Water chemistry, hydrological parameters and biotic activities influence travertine deposition (Fouke, 2011). The role of microbial mats in biomineralization processes is not fully clarified and can impact on bioremediation capabilities related to toxic metals immobilization (Kumari *et al.,* 2016; Li *et al.,* 2015). Bacterial group metabolic activity influences water chemistry and the precipitation processes (Dupraz *et al.,* 2009, Gallagher *et al.,* 2012). Thus, the knowledge on microbial species,

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their nature, distribution and functions is essential to understand travertine biomineralization and microflora establishment in hot spring waters. The microbiological component of hot spring ecosystems around the world has been studied by culture dependent and culture independent methods (López-López *et al.,* 2013; Ward *et al.,* 1998). Currently, the high-throughput sequencing methodologies (NGS, Next Generation Sequencing) can supplement the traditional culture methods, yielding a comprehensive study of complex microbial diversity by microflora DNA (mfDNA) analysis in complex matrices (Giampaoli *et al.,* 2014; White III *et al.,* 2016).
Aim of this work is to characterize the bacteria communities inhabiting Bullicame hot spring, using a cultivable and uncultivable methodology. For the first time, a comprehensive microbiota biodiversity analysis was performed on this spring, traditionally used as a natural SPA in an area rich in travertine quarries.

MATERIALS AND METHODS

Location and geochemical setting

Bullicame (N: 42° 25' 15, 78000", E: 12° 3' 52, 48800") is a thermal area located between the Tyrrhenian coastline and the Apennines, in the papal city of Viterbo (Northern Latium, Italy; *Figure 1*). The region is characterized by sedimentary rocks of upper Miocene-Pleistocene age, overlying a Paleozoic-Triassic metamorphic basement. The basins and ranges are structurally related to the extensional neotectonism that resulted in the volcanic activity in this part of Italy. The structural features of the area are very complex because of the neotectonic effects and the more recent volcano-tectonic related activities (Piscopo *et al.,* 2006). Northern Latium is characterized by a geothermal anomaly, which correlates with the structural setting on a regional scale, and in the thermal area of Viterbo, in coincidence with the structural high of the carbonate reservoir. The area includes the highest geothermal gradients, greater than 100°C/km. This area is also characterized by substantial CO_2 emissions which together with fluxes, groundwater and sedimentary rocks control, partly, the genesis of the travertine that outcrops typically around the Viterbo thermal area. In particular, the area of Viterbo (Central Italy) presents thermal waters known since ancient times and traditionally used as SPA and recreational environments like the Roman thermae. In the Middle Ages the region was also described in the "Divina Commedia", the epic poem written by Dante Alighieri, with clear references to the "Bullicame" waters (cfr, "Inferno" cantos XII and XIV). The Bullicame spring system is characterized by a Ca-SO_4-HCO_3 composition typical of most waters circulating in the mesozoic carbonate sequence.

Sampling

Water and mat samples were collected in triplicate from the same sites in November 2013. Water samples for chemical, microbiological and molecular analysis was collected by draining water from the drainage channel in front of the source, in 1-liter borosilicate sterile glass bottles. The thermal spring deposits (500 mg) covered by microbial mats and in contact with water were sampled using an aseptic scalpel and stored in sterile plastic bags in the channel. To enhance consistency and reduce local heterogeneity, each sampling was collected in triplicate from three independent points within a 20 cm x 20 cm area and then pooled together. Samples for all analyses were immediately stored on-site at 4-6°C and transferred within 3-5 hours to the laboratory for processing.

Physicochemical water characterization

Water quality parameters, namely: temperature, pH, electrical conductivity, free CO_2 were measured in situ using the relevant field meters (Mettler Toledo meters, UK and QRAE II, RAE Systems, USA) while other physical-chemical parameters were determined in the laboratory, applying the APAT 2003 Italian official methods. Briefly, laboratory analyses of the water samples were performed by atomic absorption spectrophotometry for Na^+, K^+, Mg^{2+}, Ca^{2+}, Li and Fe liquid. Hydrogen sulphide (H_2S) for the thermal waters was stabilized with zinc acetate and then determined in the laboratory by direct titration of the sulphur ion with iodine and retrotitration of the excess

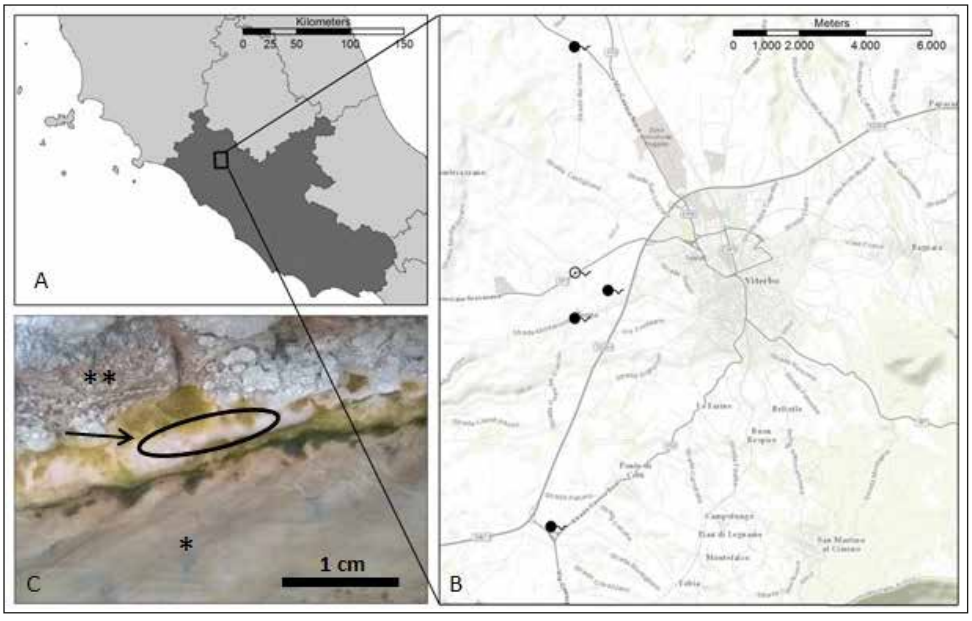

Figure 1 - *Geographical location of the Bullicame hot springs (A and B) and sampling area (C).* ⊙ *hot spring collection point;* ● *other hot springs belonging to the same hydrogeological group; * water and sediments; ** dry area; the arrow shows the selected sampling area.*

iodine with thiosulphate. Chromatography was used for SO_4^{2-} and HCl titration for HCO_3^-. Hardness and conductivity were determined according to the Italian reference method (Italian Health Institute, 2007). Many hydrogeological data, saturation indices (Calcite, Dolomite and Gypsum), minor and trace elements were obtained from the literature (Chiocchini *et al.,* 2001; Piscopo *et al.,* 2006; Di Benedetto *et al.,* 2011).

Strain isolation

Bacteria were isolated from microbial mats and water samples. Briefly, 0.5 g of lithified mats were dissolved in 10 ml medium D (Nitrilotriacetic acid 0.1 g, H_2SO_4 0.05 ml, $MnSO_4$-H_2O 0.23 g, $ZnSO_4$-$7H_20$ 0.05, H_3BO_3 0.05 g, $CuSO_4$-$5H_2O$ 0.0025 g, Na_2MoO_4-$2H_20$ 0.0025 g, $CoCl_2$-$6H_2O$ 0.0045, $FeCl_3$ 0.023 g, $CaSO_4$-2 H_20 0.06 g, $MgSO_4$-7 H_20 0.1 g, NaCl 0.008 g, KNO_3 0.103 g, $NaNO_3$ 0.689 g, Na_2HPO_4 0.111 g, 0.1% Tryptone, 0.1% Yeast extract, distiller water 1 liter, adjusted to pH 6.8. In addition 20 g/L agar for solid medium) and 500 µl aliquot spread on D medium plates (Castenholz *et al.,* 1969). Medium APL/iron-reducer was, also, used (Ogg and Patel 2009). Water samples (1 liter) were filtered with a 0.45 µm sterile nitrocellulose membrane (Whatman-GE Healthcare, USA) and incubated on D medium plates for 18 h at 54°C (other temperature and time were investigate). A total of 100 colonies were isolated and clustered in different morphotypes (e.g. size, shape, color, Gram staining) and subjected to Amplified Ribosomal DNA Restriction Analysis - ARDRA (data not shown). Strains were preserved by freezing or lyophilizing.

Strain identification

Each pure culture morphotype (n=100) was grown in 5 ml of Medium D (overnight 54°C), centrifuged and pellet lysed by GenElute Bacterial Genomic DNA Kit (Sigma–Aldrich, St. Louis, USA). Approximately 1 ng of genomic DNA was amplified in 25 µl reaction mixture consisting of 1 x Taq master mix (Promega, USA), 1 µmol l^{-1} of forward and reverse primers (8f:AGAGTTTGATYMTGGCTCAG; 1546r:CAKAAAGGAGGTGATCC; Weisburg *et al.,* 1991). The thermocycling protocol consisted of denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 15 s, 50°C for 60 s, 72°C for 90 s. Each PCR fragment arising from different morphotypes was screened for redundancies by ARDRA. Enzymatic digestion was performed by incubating 0.8-1 µg of 16S rRNA gene PCR product with 10U of restriction enzymes AluI, RsaI and TaqI (Invitrogen, Canada) in a total volume of 20 µl for 2 h 37°C. Fragments were separated by electrophoresis at constant voltage (100 V) for 45 min on 2% agarose gel. After the restriction profiles study, 30 isolates were selected for the sequencing using a BigDye Terminator cycle sequencing ready reaction kit, version 3.0 and sequencing performed on an ABI 3700 DNA sequencer (Applied Biosystems, USA). The sequences were putatively assigned to genera or species based on BLAST analysis (Altschul *et al.,* 1997). Finally, ten independent isolates were selected, biochemically characterized and their sequences deposited in GenBank (NCBI accession numbers in *Table 1*).

Phylogenetic analysis of isolated strains

The BLAST program (Altschul *et al.,* 1997) was used to compare the V1-V3 Region sequences. ClustalX v1.8 software (Jeanmougin *et al.,* 1998) was used for aligning. The phylogenetic tree was constructed using MEGA software, version 6 (Tamura *et al.,* 2013). The phylogenetic analysis was carried out by applying a neighbor-joining bootstrapping method (1,000 replicates). Sequences were aligned on partial V1-V3 hyper-variable region.

Bacterial community 16S profiling

Water (1 liter) was filtered with a 0.22 µm polyamide membrane (Sartorius, Germany), then placed in a sterile tube with 1 ml of sodium phosphate buffer plus 0.1 g glass beads (Sigma Aldrich, USA), shaken by vortex for 10 minutes, the filter removed and the suspension processed to isolate high molecular weight DNA by FastDNA®SPIN Kit (MP Biomedical, USA). In mat samples (0.500 g) DNA

Table 1 - *Putative metabolic assignment of the observed OUT in Bullicame hot springs and related references.*

Isolate		*matrix*	*Identification*	*Blast opposed sequence Accession number*	*Identity (%)*	*Accession number*	*Sequence length (nt)*
N.	*Name*						
8	2_06.05, *2B_06.05*, 162_17.04, 163_17.04, 3a_31.07, 3b_31.07, 5c_31.07, C5_31.07	*mat*	*Anoxybacillus flavithermus*	NR_117774.1	98.2	KX113649	499
2	*16_06.05*, 16b_06.05	*mat*	*Anoxybacillus contaminans*	NR_029006.1	98.4	KX113650	499
4	164_17.04, *8_06.05*, 7c_31.07, 10b_31.07	*mat*	*Anoxybacillus kamchatkensis*	NR_118117.1	98.2	KX113651	600
3	15a_31.07, *15b_31.07*, 17d_31.07	*mat*	*Bacillus licheniformis*	NR_074923.1	99.8	KX113652	549
2	*5b_31.07*, 17c_31.07	*mat*	*Bacillus subtilis*	NR_113265.1	100	KX113653	499
3	*10_06.05*, 10b_06.05, 17b_31.07	*mat*	*Aneurinibacillus danicus*	NR_114088.1	97	KX113654	498
2	*17_06.05*, 17b_06.05	*mat*	*Brevibacillus agri*	NR_113767.1	98	KX113655	499
8	*165_17.04*, 166_17.04, 167_17.04, 168_17.04, 169_17.04, 170_17.04, 171_17.04, 172_17.04	*mat/water*	*Thermomonas hydrotermalis*	NR_025265.1	98.9	KX113656	652
4	1_06.05, *1b_06.05*, 173_17.04, 1_31.07	*water*	*Tepidimonas taiwanensis*	NR_043227.1	99.6	KX113657	1303
1	*7e_31.07*	*water*	*Deinococcus murrayi*	NR_026416.1	99.2	KX113658	383

Table 2 - *Molecular identification of water and sediment isolates. 16S rRNA gene sequence nucleotide identity of cultures prepared in this work with their nearest validly described type strains.*

α-Diversity							
Sample	*S*	*N*	*Chao1*	*Chao1 (SD)*	*Shannon (H)*	*Inverse Simpson (D)*	*EH*
Water	89	8914	138.99	21.36	0.86	1.63	0.2
mat	413	51917	578.37	40.04	3.34	2.37	0.55
β-Diversity							
First Sample	*Second Sample*	*Jaccard Classic*	*Sorensen Classic*	*Chao-Jaccard-Raw Abundance-based*	*Chao-Sorensen-Raw Abundance-based*	*Morisita-Horn*	*Bray-Curtis*
Water	mat	0.215	0.355	0.973	0.986	0.433	0.293

was extracted following the same protocol. Samples were prepared according to the "16S Metagenomic Sequencing Library Preparation" guide (Part# 15044223 rev. A; Illumina, USA). The amplicon PCR has been performed using the following primers (containing overhang adapters): Ba27F 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGAGTTTGATCCTGGCTCAG-3' Ba338R 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGCTGCCTCCCGTAGGAGT-3' (Kittelmann *et al.,* 2013; Giampaoli *et al.,* 2014; Valeriani *et al,* 2017a,b; Paduano *et al.,* 2017). For Archaea community analysis, the optimal primer pair was selected after considering different primer combinations targeting the archaeal V1-V3 regions and producing amplicon length compatible with the Illumina sequencing. Libraries have been quantified by PicoGreen dsDNA quantitation assay (Thermo Fisher Scientific, USA) and validated on Bioanalyzer DNA 1000 chip (Agilent, USA). Sequencing was performed on MiSeq desktop sequencer (Illumina), following the manufacturer's protocol.

Bioinformatic analysis

The sequence reads were analyzed in BaseSpace through the 16S Metagenomics app (version 1.0.1; Illumina®): the taxonomic database used was the Illumina-curated version (May 2013 release of the Greengenes Consortium Database) (Wang *et al.,* 2013). Community richness and microbial biodiversity were computed using EstimateS software (Colwell *et al.,* 2012). For α-diversity Chao 1 index, Shannon's (H) and Simpson's inverse diversity and evenness (E_H) have been calculated While for β-diversity Classical Jaccard and Sorensen and abundance-based b-diversity measures, including Bray–Curtis and Morisita-Horn index have been reported (Magurran, 2013).

Functional profile prediction

For each single genus a putative metabolic capability was assigned, according to data reported in the literature (*Table 2*). The groups of metabolic capability were assigned by classification reported by Dupraz and Visscher (2005). Briefly, these authors classified the microbes involved in calcium carbonate precipitation and dissolution into several major groups: anoxygenic phototroph bacteria (ANOX PHOT); sulfur-reducing bacteria (SRB); sulfur oxidizing bacteria (SOB); oxygenic phototroph bacteria (OX PHOT); anaerobic heterobacteria (ANAER HETER); aerobic Heterotroph bacteria (AER HETER); fermentative and other chemiolitoauthotroph bacteria.

RESULTS

On the basis of its chemical composition, Bullicame thermal spring water was classified as sulfate bicarbonate containing alkaline earth metals, according to previous papers (Piscopo, 2006). Indeed, Bullicame hot spring water showed a hardness above 17.8 ppm $CaCO_3$, and a sulfur compounds: SO_4 1181 mg l^{-1}, H_2S 9.6 mg l^{-1}. The water (54°C, pH 6.5) conductance was 2590 µS cm^{-1}, with a Ca^{2+} level of 341 mg l^{-1}, K^+ 42 mg l^{-1} and Mg^{2+} 125 mg l^{-1}, Na^+ 35 mg l^{-1}, Li 0.19 mg $l^{-1}$and Fe 0.6 mg l^{-1}. HCO_3 840 mg l^{-1} and CO_2 free 871 mg l^{-1}. The data on traces elements were collected from the literature: Mo 0.04 ppm, Cu 0.35 ppm, Pb 0.49 ppm, Zn 1.1 ppm, Mn 121, As 85.5, Th 0.3 ppm, Cd 0.03 ppm, Sb 0.03 ppm. Other trace elements were below the operative detection limit (i.e. Hg <10 ppb; Ag, Au <40 ppb; Tl <0.02 ppm; Bi <0.04 ppm; Co, U, W, Se <0.2 ppm; Ni, Ga, Sc <0.6 ppm; V <2 ppm; La <3 ppm; Ti <10 ppm, B <50 ppm.). In addition, saturation indices for calcite, dolomite and gypsum species for water sample were collected from the literature: these waters are slightly sub-saturated with respect to Gypsum, with positive values of calcite and dolomite (0.66 and 1.18, respectively), and a negative value of Gypsum (-0.31; Piscopo *et al.,* 2006).

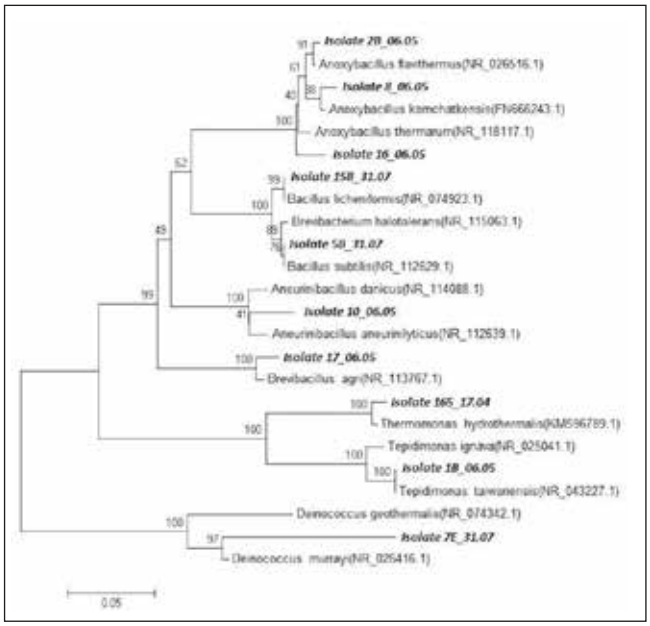

Figure 2 - *Neighbor-joining distance-based phylogenetic tree based on partial V1-V3 region of 16S rRNA gene sequences. Percentage bootstrap values for 1000 trees are shown at branch points with only values of 40% or greater included. GenBank database accession numbers for known micro-organisms are indicated. The phylogenetic tree reports the isolated strains and their nearest neighbor.*

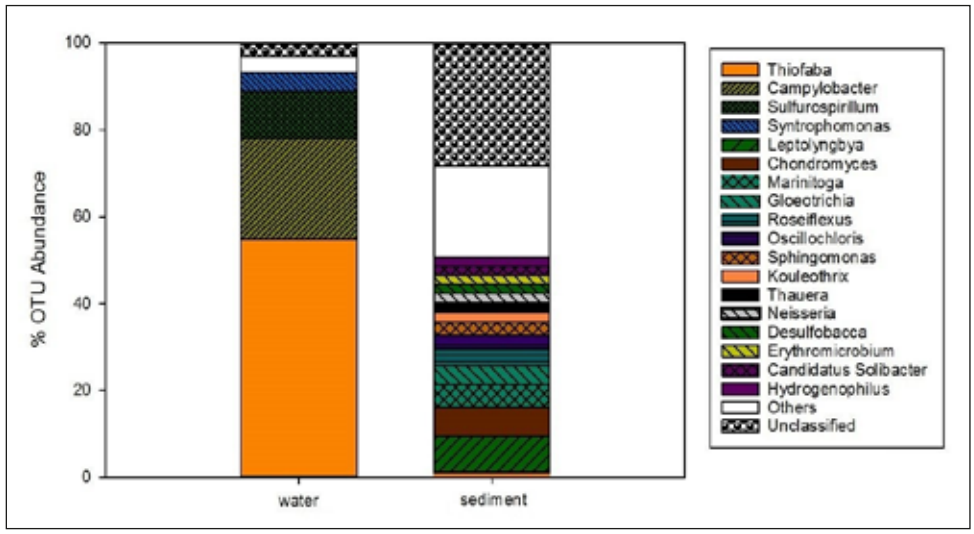

Figure 3 - *OTU abundance at genus level in water and mat of Bullicame hot springs. The percentage of occurrence of each taxon is reported as a cumulative bar chart. The legend shows the list of taxa from top to bottom of the bars. Thiofaba (54%) is the genus prevalent in water, whereas in mat there is greater biodiversity.*

Starting from one liter of water, it was possible to obtain cultivable aerobic bacteria on medium D modified plates after an overnight incubation at 54°C: 1.36×10^5 CFU ml^{-1} in water and 5×10^5 CFU g^{-1} in mats. Fewer colonies were present on medium APL/iron-reducer. Morphology selected isolates (n=37 out of a total of 100 colonies) were classified by 16S rDNA alignment allowing the identification of 10 independent species (*Table 1*). The putative species detected in mats included: *Anoxybacillus*, *Bacillus*, *Aneurinibacillus*, *Brevibacillus agri*. *Thermomonas hydrotermalis*, was collected from both water and mat, whereas *Deinococcus murrayi* and *Tepidimonas taiwanensis* only from water. The neighbor-joining phylogenetic tree (*Figure 2*) reports the isolated strains: *Firmicutes*

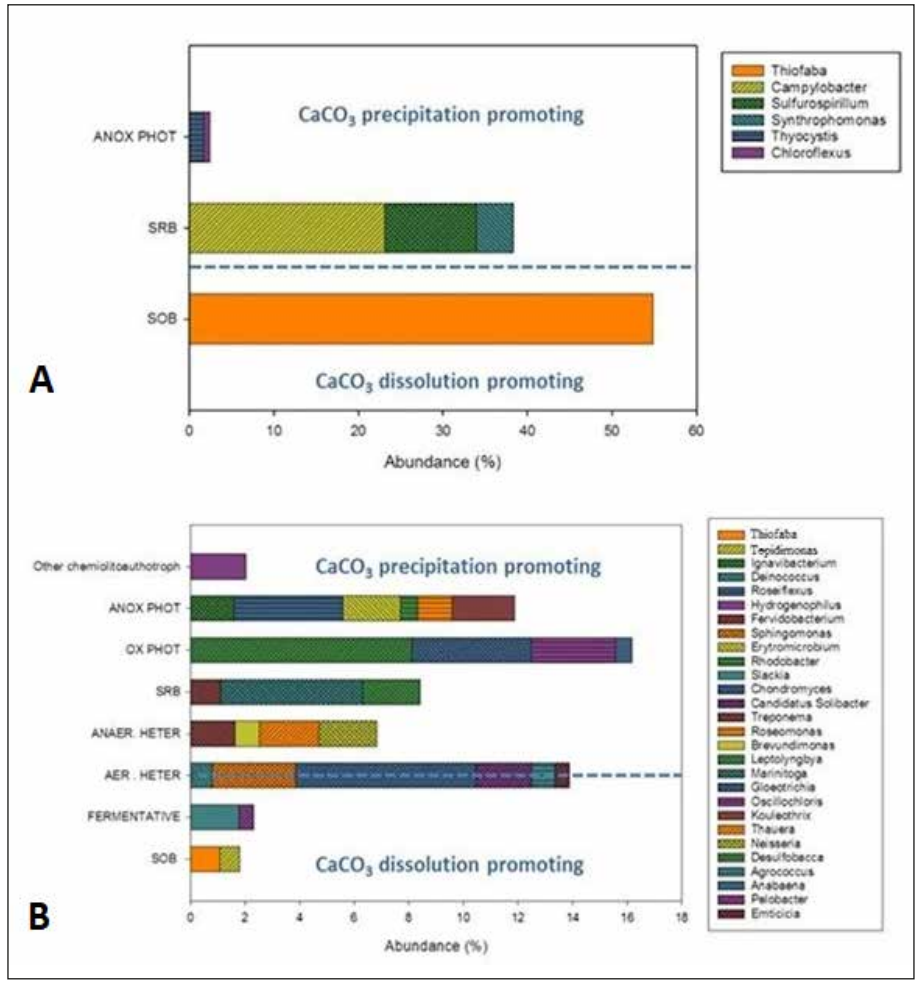

Figure 4 - *Overview of the putative impact of OTU involved in $CaCO_3$ precipitation or dissolution. The functional groups are reported in graph as abbreviations: anoxygenic phototroph (ANOX PHOT); sulfur-reducing (SRB); sulfur oxidizing bacteria (SOB); oxygenic phototroph (OX PHOT); anaerobic heterobacteria (ANAER HETER); aerobic Heterotrophs (AER HETER); Fermentative and other chemiolitoauthotrophs are also indicated. The abundance of each taxon is reported as a cumulative bar chart with the percentage value. A) In water $CaCO_3$ dissolution is promoted by SOB bacteria (Thiofaba) whereas $CaCO_3$ precipitation is supported by SRB bacteria (Campylobacter, Sulfurospirillum, Syntrophomonas), ANOX PHOT (Thiocystis, Chloroflexus). B) In mats the abundance of each genus associated by $CaCO_3$ dissolution is reported as SOB bacteria (Thiofaba, Tepidimonas), fermentative bacteria (Slackia, Chondromyces), whereas $CaCO_3$ precipitation is promoted by SRB bacteria (Marinitoga, Desulfobacca and Fervidobacterium), ANAER HETER (Thauera, Neisseria), OX PHOT (Leptolyngbya, Gloeotrichia and Oscillochloris), ANOX PHOT (Roseiflexus, Kouleothrix, Erythromicrobium, Ignavibacterium), and other chemiolitoauthotroph bacteria (Hydrogenophilus).*

(66%), *Proteobacteria* (25%), *Deinococcus-Thermus* (1%). Therefore, we applied an NGS approach to further study the composition of the bacterial community in water and mats, obtaining 31,497 and 113,617 high-quality sequences, respectively. Rarefaction curves were calculated for both samples (data not reported), showing the achievement of a plateau. Sequence analysis provided a total of 11 bacterial phyla. In water, the main phyla were: *Proteobacteria* (91%), *Firmicutes* (5%), *Chloroflexi* (1%) and other categories (<1%) including *Thermi*, *Bacteroidetes*, *Chlorobia*, *Actinobacteria* and unclassified phyla (2%). In the lithified microbial mats sample, *Proteobacteria* (40%), *Cyanobacteria* (13%), *Chloroflexi* (11%), *Firmicutes* (7%), *Thermotogae* (6%), *Bacteroidetes* (3%), *Acidobacteria* and *Chlorobi* (2%) were the observed phyla, in addition to unclassified (10%) and other categories (6%) such as *Actinobacteria*, *Spirochaetes* and *Thermi*. The Archaea community represented only 0.2% in water and mat samples. However, even if it is important remember that the approach we used is wide and not specific for detailed analysis, this particular subgroup and a specific analysis highlighted the presence of the main phyla, such as Euryarchaeota and Crenarchaeota. The diversity and distribution of the bacterial Operational Taxonomic Units (OTUs) was defined at genus taxonomical level and identified in the metagenomic analysis from water and microbial mats of the hot spring. A summary is presented in *Figure 3*. The water sample is dominated by *Thiofaba* (55%), followed by *Campylobacter* (23%), *Sulfurospirillum* (10.8%), *Syntrophomonas* (4.4%) and other genera with percentages less than 1% (total 3.7%). The unclassified sequences at genus level were 3.1%. In the microbial sediment sample the genera most represented are: *Leptolyngbya* (8%) and *Chondromyces* (7%) followed by *Marinitoga* (5%), *Gloeotrichia* and *Roseiflexus* (4%), *Oscillochloris* and *Sphingomonas* (3%), and *Desulfobacca, Kouleothrix, Thauera, Neisseria, Erythromicrobium*, *Candidatus Solibacter, Hydrogenophilus* (2%), *Thiofaba* (1%). Other less represented genera were found in less than 1% (in total 23%), including *Tepidomonas* and *Deinococcus*;

Table 3 - *Biodiversity indexes. α-Diversity indices. Number of species for sample (S), Number of individuals (N), Chao1, Chao1 standard deviation (SD), Shannon (H), Inverse Simpson (D) and Evenness (E_H) of the bacterial communities in hot spring for water and sediment; β-Diversity indices calculated in water and sediment samples based on NGS data. Jaccard Classic, Sorensen Classic, Chao-Jaccard-Raw Abundance-based, Chao-Sorensen-Raw Abundance-based, Morisita-Horn, Bray-Curtis.*

Genus	*Metabolic classification*	*References*
Thiofaba	Sulfur-oxidizing	Mori and Suzuki 2008
Campylobacter	Microaerofilic heterotroph	Wagley *et al.*, 2014
Sulfurospirillum	Sufur-reducing	Sikorski et al., 2010; Kodama *et al.*, 2007
Syntrophomonas	Anaerobic heterotroph	Wu et al., 2006; Souza *et al.*, 2007
Thyocystis	Anoxigenic phototropic/sulfur oxidizing,	Peduzzi *et al.*, 2011
Chloroflexus	Phototrophic anoxigenic	Tang *et al.*, 2011; Gaisin *et al.*, 2017
Tepidimonas	Aerobic heterotroph	Chen *et al.*, 2013
Ignavibacterium	Photoautotroph /fermentative	Liu *et al.*, 2012
Deinococcus	Heterotroph aerobic	Makarova *et al.*, 2001
Roseiflexus	Anoxigenic phototroph	van der Meer 2010
Hydrogenophilus	H2 oxidizing/heterotrophic	Vésteinsdóttir *et al.*, 2011
Fervidobacterium	Fermenting	Podosokorskaya *et al.*, 2011; Cai *et al.*, 2007
Sfingomonas	Aerobic Heterotrophs	Gan *et al.*, 2014
Erythromicrobium	Phototrophic anoxigenic	Yurkov and Beatty 1998
Rhodobacter	Anoxigenic phototroph /chemoheterotroph	Boran *et al.*, 2010; Imam *et al.*, 2011
Slackia	Fermenting	Nagai *et al.*, 2010; Jin *et al.*, 2010
Chondromyces	Aerobic Heterotroph	Zaburanny *et al.*, 2016
Candidatus Solibacter	Aerobic Heterotroph	Challacombe *et al.*, 2011
Treponema	Anaerobic Heterotroph	Radolf and Lukehart 2006
Roseomonas	Anaerobic heterotroph	Klann *et al.*, 2016
Brevundimonas	Aerobic Heterotroph	Abrham *et al.*, 2010
Leptolyngbya	Photoautotrophic oxigenic	Shimura *et al.*, 2015
Marinitoga	Sulfur reducing/anaerobic heterotroph	Alain *et al.*, 2002; Lucas *et al.*, 2012
Gloeotrichia	Anoxigenic phototroph	Whitton *et al.*, 2012
Oscillochloris	Anoxigenic phototroph	Kuznetsov *et al.*, 2011; Keppen *et al.*, 2015
Kouleothrix	Oxigenic phototroph	Li *et al.*, 2017
Thauera	Anaerobic heterotroph	Jiang *et al.*, 2012
Neisseria	Aerobic heterotroph	Baart *et al.*, 2007
Desulfobacca	Sulphate reducing	Goker *et al.*, 2011; Stams *et al.*, 2015
Agrococcus	Aerobic heterotroph	Kämpfer and Busse 2015
Anabaena	Phototrophic	Curatti *et al.*, 2002
Pelobacter	Sulfur reducing	Purdly *et al.*, 2003
Emticicia	Aerobic heterotrophic	Saha and Chakrabarti, 2006

28% of the total bacterial 16S rRNA gene sequences were not classifiable. *Figure 4* gives an overview of the putative impact of genera involved in $CaCO_3$ precipitation or dissolution. Data collected in the literature were used to assign the putative metabolic capability of the each genera obtained by NGS (*Table 3*) and classify them in the adequate functional groups. *Figure 4A* reports the abundance of each genus as cumulative bar chart with the percentage value for water: $CaCO_3$ dissolution is promoted by SOB bacteria, as *Thiofaba* genus (55%), whereas $CaCO_3$ precipitation is reported by SRB bacteria, such as *Campylobacter* (23.1%), *Sulfurospirillum* (10.8%), Syntrophomonas (4.4%), and ANOX PHOT, as Thiocystis (1.7%), Chloroflexus (0.8%). *Figure 4B* reports the abundance of each genus as a cumulative bar chart with the percentage value for mat: $CaCO_3$ dissolution is promoted by SOB bacteria, as *Thiofaba* genus (1%) and *Tepidimonas* (0.7%), or fermentative bacteria, as *Slackia* (1.77%); *Chondromyces* (6.6%), whereas $CaCO_3$ precipitation is reported by SRB bacteria, such as *Marinitoga* (5.2%), Desulfobacca (2.1%) and *Fervidobacterium* (1.1%), ANAER HETER, *as Thauera* and *Neisseria* (2.1%), and OX PHOT, as *Leptolyngbya* (8.1%), *Gloeotrichia* (4.4%) and *Oscillochloris* (3%). Moreover, ANOX PHOT, as *Roseiflexus* (4%), *Kouleothrix* (2.3%), *Erythromicrobium* (2%), *Ignavibacterium* (1.6%), and other chemiolitoauthotroph bacteria, such as *Hydrogenophilus* (2%) also participated in the $CaCO_3$ precipitation process. Biodiversity α and β indices were analyzed (*Table 3*).

The Shannon index was 3.34 in mat and 0.86 in water sample while the heterogeneous distribution of the relative abundances of species, represented by evenness index, was 0.55 and 0.2, respectively in sediment and water. Analyzing the β-diversity values, Sorensen, Jaccard classic and Bray-Curtis showed the following values 0.215, 0.355 and 0.293, respectively. Moreover, the Morisita-Horn score was 0.433.

DISCUSSION

Several chemical-physical components can influence bacteria biodiversity in hot springs. In extreme environments the presence of limiting factors (e.g. toxic compounds for microorganisms, high/low pH, low amount of nutrients, low amount of oxygen) create a selective environment where pioneer communities are able to live and shape the ecosystem (Gagliano *et al.,* 2016; Chiriac *et al.,* 2017). Specifically, the impact of biotic and abiotic pathways on travertine deposition plays a major role in microbial ecology and in the final composition of the waterborne microbiota. Metabolism of some bacterial groups influences water chemistry, favoring the carbonate precipitation processes (Dupraz and Visscher, 2005). The role of microbial mats in mineral precipitation processes is not fully clarified, and the identification of the key factor shaping the water and sediment communities is needed. For the first time, this work studied the specific characterization of the bacteria communities inhabiting Bullicame hot spring, using a cultivable and uncultivable methodology to investigate the key factor shaping the water and sediment communities. According to previous studies, hot springs are characterized by a reduced biodiversity comparing to other aquatic environments (Kemp and Aller, 2004; Chiriac *et al.,* 2017). This aspect was clearly underlined by the bacterial recovery described in this work, in which only low bacteria concentrations per liter of hot spring water were detected. A possible explanation for this phenomenon can be related to a bacteriostatic effect of hydrogen sulfide and other chemical compounds dissolved in volcanic hot spring waters (Giampaoli *et al.,* 2013). Moreover, this spring continuous flow recharges the water columns with nutrients, changes the water composition, in all probability continuously varying the water ecosystem (Piscopo *et al.,* 2006). This condition could create an environment more suited to pioneer/resistant species translated in a low bacterial diversity. Indeed, the genera identified by the cultural method were in most cases *Firmicutes*, including spore-forming bacteria, very resistant to chemical and physical conditions. However, pioneer species such as *Tepidimonas* or *Deinococcus* were recovered in this water. In particular, *Tepidimonas taiwanensis* strain VT154-175 was isolated, the draft genome of which was described elsewhere (Valeriani *et al.,* 2016). On the other hand, it is more difficult to vary the chemically more stable sediment composition and the bacterial community could have more steady conditions to create microniches leading to a higher diversity (Bolhuis *et al.,* 2014). The described microbial communities could to be linked to different parameters including fluid hydrodynamics, chemical-physical properties and hydrogeological contexts.

In this work, culture methods allowing investigations *in vivo* were flanked by studies on complex microflora structure by molecular technologies that yielded information on other viable but uncultivable species (López-López *et al.,* 2013; Mansi *et al.,* 2014). A multidisciplinary study implementing both cultural and molecular methods has been developed and can support a complete overview of the ecosystem. By NGS analysis different bacterial phylotypes were recovered and successfully characterized. In the NGS analyses several high-quality sequences were obtained and the differences between water and mat outputs were in line with previous papers (Badhai *et al.,* 2015; Song *et al.,* 2013). Some genera seem to match both in sediments and in water, even in water with lower concentrations, such as *Thiofaba* (55 vs 1%), *Campylobacte*r (23 vs 0.4%), *Sulfurospirillum* (11 vs 0.23) and *Syntrophomonas* (4 vs 0.07%). Moreover the bacteria heterogeneity was defined by α and β diversity (*Table 3*). The Shannon index showed a higher biodiversity (H'=3.34) in mats respect to water samples (H'=0.86). The decrease of α diversity in water is not surprising and is in line with previous works in the literature (Lau *et al.,* 2009; Tobler and Benning, 2011; Chiriac *et al.,* 2017). Moreover, a heterogeneous distribution of species abundance (Evenness Index=0.55) was observed in the mat with respect to water. In increasing biodiversity and in diversified mat the phylum level was also observed. In particular, *Proteobacteria* phylum prevailed in both water and mat samples, but in water all other categories have low percentages, with the exception of *Firmicutes*. The phyla level investigation also showed a very high number of unclassified phyla (10%). This is probably due the poor known field of extremophiles and thermal microorganisms, that is still not so rich in databases as for other microorganisms and microbial communities (e.g. human microbiota).

The analysis was also deepened at a genus level, showing very different insights. *Thiofaba* genus was prevalent in water (54%). It comprises obligate chemolithoautotrophic sulfur-oxidizing bacteria utilizing H_2S as electron donor for CO_2 reduction (Van Gemerden, 1993). The role of sul-

fur-oxidizing bacteria is crucial as biotic factors affecting carbonate equilibrium and promoting carbonate dissolution (*Figure 4*). Conversely, *Campylobacter*, *Sulphirspirillum* and *Syntrophomonas* comprise lithoautotrophic sulfur-reducing bacteria, promoting $CaCO_3$ precipitation and represent the 38% of the water community. This microbial configuration is typical of oligotrophic environments (Phelps *et al.,* 1994). Mats showed a more heterogeneous structure with an increase in those heterotrophic genera that may probably act in capturing and metabolizing organic matter (Pentecost 2005; Schubotz *et al.,* 2015). A high occurrence of filamentous cyanobacteria, such as *Leptolyngbya, Oscillochloris, Gloeotrichia,* was observed. Besides their role in fixing CO_2, they are likely to be the main architects of stromatolites by trapping and binding sediments in the adhesive matrix of exopolymeric substances, finally ending in carbonate precipitation (Reyes *et al.,* 2013). All the functional groups were observed in mats, including: oxygenic phototrophs, mainly represented by cyanobacteria, e.g. *Leptolyngbya*, which are involved in CO_2 fixation and crystal nucleation (Dupraz *et al.,* 2005; Saini *et al.,* 2011); anoxygenic phototrophs, e.g. *Roseiflexus* and *Roseomonas*; sulfur-reducing bacteria e.g. *Marinitoga* and *Desulfobacca*, and anaerobic heterobacteria, e.g. *Nesseira, Thauera and Treponema*. All these genera are well represented and linked to $CaCO_3$ precipitation. Otherwise, OTUs related to dissolution processes were detected in lower abundance (<5%), including fermentative bacteria, e.g. *Slakia* and *Pelobacter* and sulfur oxidizing bacteria e.g. *Thiofaba* and *Tepidimonas*. Microbial components may also influence pH, thus affecting the saturation index (Gallagher *et al.,* 2012). Bacterial metabolism drives the alkalinity engine, which subsequently generates a microenvironment where microbialites facilitate carbonate precipitation (Dupraz *et al.,* 2005). In this equilibrium, a fundamental contribution to the alkalinity engine is associated with sulfur-reducing bacteria, e.g. *Marinitoga* and *Desulfobacca*.

The Bullicame hot spring represents a unique extreme environment with specific physical-chemical properties. Considering the alkaline characteristics of the water and the high concentration of Sulfur species and HCO_3 (Piscopo *et al.,* 2006; Giampaoli *et al.,* 2013), this hot spring represents an ideal site for studying travertine deposition, since sulfur transformations strongly affect dissolution and precipitation processes. In this environment, microorganisms produce biofilms that consist of different bacteria, products of their metabolism, sedimentary particles and organic matter that induce the development of a benthic microflora. These processes are supported by water proprieties such as an elevated Saturation Index for calcite and dolomite and slight sub-saturation with respect to gypsum in the presence of a gas phase dominated by CO_2 (Di Benedetto *et al.,* 2011). Probably, the continuous water flow, the exposure to light and the lack of disturbance due to human activities led in time to microbial mats presenting a layered structure. Benthic microbial communities play a major role in carbonate precipitation, assuming that travertine deposition is not purely a consequence of degassing (Rogerson *et al.,* 2008). Therefore, knowledge on benthic and planktonic bacterial communities in hot springs acquires a particular significance. Although it is strongly recognized that microorganisms play a role in calcium carbonate precipitation, there is a lack of information to clarify the effort of biotic and abiotic factors affecting CO_2 sequestration (Okyay *et al.,* 2015). A preliminary study carried out on Bullicame hot springs assumed that abiotic gas evasion is the major driving force in CO_2 sequestration (Pentecost, 2005). Interactions among functional microbial groups and a wide range of environmental conditions can clarify the role of mats in biomineralization and water biodiversity, requiring knowledge on both microbiota and metabolic activities (Dupraz *et al.,* 2005, 2009).

In conclusion, Bullicame spring water is characterized by a complex bacterial microflora. Mats contribute to define the water microbiota through different processes. Chemical-physical conditions influence the microbial community but mats microbialite also play a role in maintaining biodiversity and inducing biomineralization (Van Gemerden, 1993; Decker *et al.,* 2005; Dupraz *et al.,* 2005 and 2009). The application of NGS analysis provides a powerful contribution in characterizing microbial communities in these natural environments, providing information on natural microflora and metabolic potentials. Our results describe factors, such as $Ca\text{-}SO_4\text{-}HCO_3$ composition, that could determine the formation of these ecosystems, expanding current knowledge in this regard. In this perspective, the Bullicame thermal spring water could be characterized not only for its chemical and physical parameters but also for its microbiota structure, appearing as a "biological fluid" with active properties. This paper studied the bacterial community present in the Bullicame hot spring water relating it to some environmental conditions. Analysis of water and mats mfDNA allowed a more comprehensive understanding of the microbial community, supporting a role for photosynthetic and sulfur reducing groups in carbonate precipitation and natural biofilm establishment. The analysis of hot spring microbiota may open further horizons for environmental protection of these natural resources and exploitation of the SPA properties known since ancient times.

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Conflict of interest statement

No conflict of interest declared

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